Bound Ligand Motion in Crystalline Carboxypeptidase A

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ABSTRACT Deuterium NMR spectra for the phenyl ring deuterons have been obtained for D-phenylalanine, L-phenylalanine, phenylacetic acid, and phenyl propionic acid in randomly oriented crystals of carboxypeptidase A as a function of water content. The spectra are analyzed using a two-site jump model for phenyl ring π-flips when the ligand is bound to the protein, and the model includes the possibility that the ligand may exchange with isotropic or unbound environments within the crystal. Although the binding pocket may impose local dynamical constraints, a complete π-flip motion is consistent with the spectra of all ligands at all water contents. The rate constants for the π-flip at 298 K are found to be 7.5×10^5 s^{-1}, 1.9×10^6 s^{-1}, 4.0×10^6 s^{-1}, and 4.0×10^6 s^{-1} for L-phenylalanine, D-phenylalanine, phenyl propionic acid, and phenylacetic acid, respectively, at water activity of 0.98. The π-flip rate for the ligand bound to the enzyme increases with water content. Assuming that the activation barrier may be written, $\Delta G^{\ddagger} = \Delta G^{\ddagger\circ} + ba_w$, where a_w is the water activity, and the value of b is -1.9 kcal/mol for phenylacetic acid and phenyl propionic acid, -1.3 kcal/mol for L-phenylalanine, and -2.1 kcal/mol for D-phenylalanine. Phenylacetic acid crystals were studied as an example of a phenyl ring motion that is highly constrained by a known and symmetrical packing environment. The deuterium spectra are complex and are not consistent with π-flip motions, but they are consistent with a superposition of ring jump motions of 24°, 34°, and 72°, with probabilities in the ratio of 1:1:2. Because of the limited space for motion imposed by the tight packing in the crystal, these motions must be highly cooperative and probably locally coherent; however, the spectra by themselves do not prove this intuitively reasonable hypothesis.

INTRODUCTION

There is considerable structural information available on enzyme active sites and substrate molecules bound in them; however, the connection between the structural information and the thermodynamics or kinetics of the enzyme-ligand interaction remains incomplete. One feature missing from the information currently available is a good picture of the motions available to the enzyme-bound ligand. Magnetic resonance methods may provide dynamical information over a very wide range of frequencies, depending on the particular measurement made. The local dynamics of a small molecule may be clearly detected in the case in which the protein is rotationally immobilized, which suppresses the rotational averaging of inherently anisotropic interactions such as chemical shift or quadruple coupling. Deuterium NMR has been very successfully applied to the characterization of molecular dynamics in lipid systems and engineering polymers (Spiess and Sillescu, 1981; Torchia and Szabo, 1982; Smith and Oldfield, 1984; Spiess, 1985; Schmidt et al., 1986, 1988; Hiyama et al., 1986; Hirschinger and English, 1989; Wefing and Spiess, 1989; Wefing et al., 1989; Schafer et al., 1990; Hirschinger et al., 1991; Vold and Vold, 1991; Spiess, 1991). The deuterium lineshape is very useful for examining motions in the time range from microseconds to milliseconds, and the use of various relaxation times may extend the range from nanoseconds to approximately a second. Our approach is to examine the deuterium NMR lineshape and spin relaxation rates of deuterium-labeled ligands bound in enzyme active sites to determine the nature of dynamical constraints imposed on the bound ligand and how the dynamics change with modifications in the structure of the ligand or with perturbations of the protein.

Carboxypeptidase A is an extensively studied enzyme for which there are a great deal of structural data (Steitz et al., 1967; Lipscomb et al., 1970; Christianson et al., 1989; Yun et al., 1992; Mustafi and Makinen, 1994). This protein provides a well-formed hydrophobic pocket into which aromatic rings fit as part of the substrate-binding mode (Lipscomb et al., 1970; Yun et al., 1992). In a previous study we found that the NMR lineshape of phenyl ring deuterons of L-phenyl alanine bound to carboxypeptidase A in the crystal may be interpreted by using a dynamic model that included rapid π-flip motions and exchange between the active site and unbound approximately isotropic environments in the crystal (Zhang and Bryant, 1995). In the present work we examine how the bound ligand dynamics are altered when the ligand position in the active site changes in response to structural differences in the bound ligand. We also examine phenyl ring dynamics in crystalline phenylacetic acid, which raises interesting questions about the nature of motions in highly constrained environments.

Received for publication 16 July 1996 and in final form 11 October 1996.

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0006-3495/97/01/363/10 $2.00

MATERIALS AND METHODS

Carboxypeptidase A (Allen type, from Sigma Chemical Co., St. Louis, MO) was crystallized and crossed-linked by the method described previously (Zhang and Bryant, 1995). The enzyme-ligand complexes were obtained by soaking the cross-linked crystals in 20 mM (L-Phe) or 10 mM

(D-Phe, PPA, PAA), and 20 mM Tris buffer solution at pH 7.4 for a week. These loaded cross-linked crystals were rinsed three times with 20 mM Tris buffer at pH 7.4, the supernate was discarded, and the wet crystals were dried under a mechanical vacuum for 30 min. The sample was then placed in a closed jar for a week over saturated solutions of calcium chloride hexahydrate, ammonium sulfate, or copper sulfate pentahydrate to establish the equilibrium water content of the crystals.

L-Phenylalanine-d5 was purchased from Cambridge Isotope Laboratories (Woburn, MA). D-Phenylalanine-d5, phenylacetic acid-d5 (PAA), and phenylpropionic acid-d5 (PPA) were synthesized by the method of Moss and Schoenheimer (1940). These ligands have reported affinity constants of 18 mM (L-Phe), 2 mM (D-Phe), 0.39 mM (PAA), and 0.062 mM (PPA). The optical purity of D-Phe-d5 was found to be 85%, based on optical rotation after the deuteration procedure; however, the binding constants imply that the concentration of bound L-Phe-d5 will be less than 2%.

Phenylacetic acid forms monoclinic crystals: $P2_1/a$, a = 10.201 Å, b = 4.9568 Å, c = 14.437 Å, β = 99.17°, Z = 4, as initially solved in the pioneering work of Patterson (Hodgson and Asplund, 1991). Hodgson and Asplund redetermined the structure in 1991, which shows that the interlayer spacing is 4.95 Å between carbon atom centers, and the ring overlap area is approximately 80% (Manojlovic and Speakman, 1968; Hodgson and Asplund, 1991). Thus the molecular packing is very tight, leaving little room for motion such as π-flips of the phenyl rings. Because the quality of the crystals may be affected by the rate of crystallization, we examined powder diffraction patterns for crystals recrystallized slowly at room temperature and those recrystallized rapidly by thermal quenching using a Scintage diffractometer (Scintage, Santa Clara, CA) at a wavelength of 1.54 Å (Cu, Kα); 2θ varied from 5° to 70°, and the chopper increment was 0.03°. These data indicated no significant difference in the diffraction patterns of the crystals prepared by these two methods.

All NMR experiments were performed with a Varian Unity-Plus NMR spectrometer (Varian Associates, Palo Alto, CA) operating at 76.6 MHz for deuterium with a home-built probe that employed a 7-mm solenoidal coil positioned perpendicular to the static field, which had a typical 90° pulse width of 2.8 μs using the low band 300-W rf amplifier. For carboxypeptidase A-ligand complexes, the solid-echo sequence used an echo delay of 35 μs and recycle delay times between 0.5 s and 2 s. Longer recycle delays did not improve the spectrum. The pulse sequence used for the two-dimensional solid ^{2}H NMR exchange spectra was $(90)_x$-t_1-$(90)_{x'}$-τ_{mix}-$(90)_x$-τ-$(90)_y$-τ-acquire, with a mixing time of 0.4 ms to 4 ms, an echo delay of 35 μs, and a dwell time of 2 μs, with 80 real × 1024 complex points, and zero filling in the t_1 domain up to 4096 points. For phenylacetic acid, the solid-echo sequence used echo delays of 35 μs and 150 μs, and the recycle delay was 100 s. Temperature was controlled with a Lakeshore DRC-82 controller, which provided a temperature-controlled gas stream that impinged directly on the sample and rf coil.

The numerical simulations of the deuterium NMR spectra were made using modifications of the programs SPOWDER (Wittebort et al., 1987) and the numerical routine for two-site exchange in heterogenous phases from the program QEXCH (Zhang and Bryant, 1995). If we turn off reorientation, this program is equivalent to QEXCH; if we turn off the chemical exchange effects in addition, it is equivalent to SPOWDER. The simulation used 2048 points, with a line broadening of 1 kHz.

RESULTS

Deuterium NMR spectra for L-phenyl alanine, D-phenylalanine, phenyl propionic acid and phenylacetic acid deuterated in the ring positions and perfused into carboxypeptidase A crystals, which were cross-linked with glutaraldehyde, are shown in Figs. 1–4. The spectra may be simulated by including both bound site motions of the phenyl ring, which we model as π-flips, and exchange motions between the bound environment and the more isotropic spaces between protein molecules in the crystal. The kinetic parameters that correspond to the computed spectra are summarized in Table 1. The spectra fall in a regime in which chemical exchange between the active site anisotropic and the isotropic environment is required. A change in this rate constant by a factor of 2 changes the simulated spectrum significantly (Zhang and Bryant, 1995). The values of the phenyl ring π-flip rates obtained from the simulations increase with water content with each bound molecule studied by a factor between 2 and 8. Fig. 5 shows that

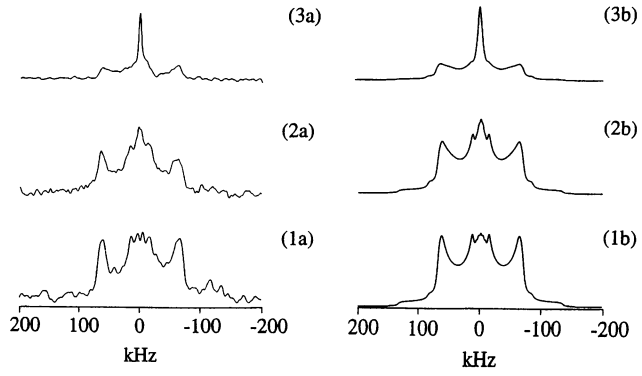


FIGURE 1 ^{2}H NMR spectra of polycrystalline carboxypeptidase A samples cross-linked with glutaraldehyde and soaked in a 20 mM solution of L-Phe-d5 obtained at 11.7 T at different water contents. Spectra were obtained using approximately 200-mg samples, 60,000 to 128,000 transients, and a deuterium quadrupole-echo delay of 35 μs: (*1a*) 6% water, 2-s recycle delay; (*2a*) 11%, 2-s recycle delay; (*3a*) 21%, 0.5-s recycle delay. The corresponding simulated spectra were obtained assuming a π-flip motion for bound ligand, a quadrupole coupling constant of 180 kHz, η = 0.05, line broadening of 1 kHz, and 2048 points in the spectral array. The exchange rate constant, $k_2 = 1.6 \times 10^4$ s^{-1}, the concentrations, C_i, and π-flip rate are: (*1b*) C_{ES} = 0.9, CS = 0.1, $k_1 = 1.9 \times 10^5$ s^{-1}; (*2b*) C_{ES} = 0.85, CS = 0.15, $k_1 = 5.0 \times 10^5$ s^{-1}; (*3b*) C_{ES} = 0.7, CS = 0.3, $k_1 = 7.5 \times 10^5$ s^{-1}.

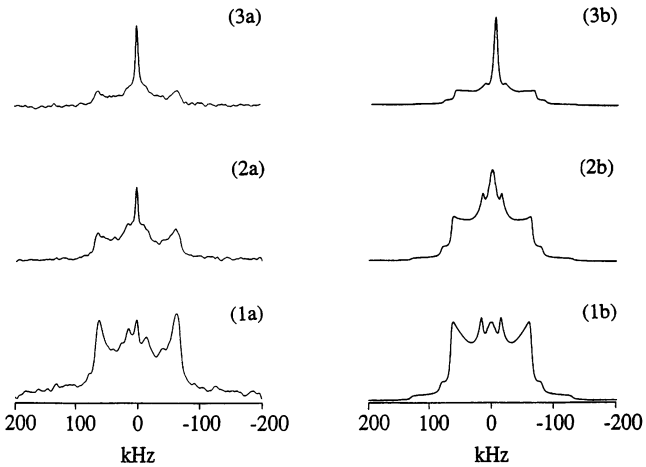


FIGURE 2 ^{2}H NMR spectra of polycrystalline carboxypeptidase samples cross-linked with glutaraldehyde and soaked in a 10 mM solution of D-Phe-d5 obtained at 11.7 T at different water contents. The experimental conditions and the approach to the simulations were the same as in Fig. 1, except that $k_2 = 1.1 \times 10^4$ s^{-1} and (*2b*) $k_1 = 1.5 \times 10^6$ s^{-1}, and (*3b*) $k_1 = 1.9 \times 10^6$ s^{-1}.

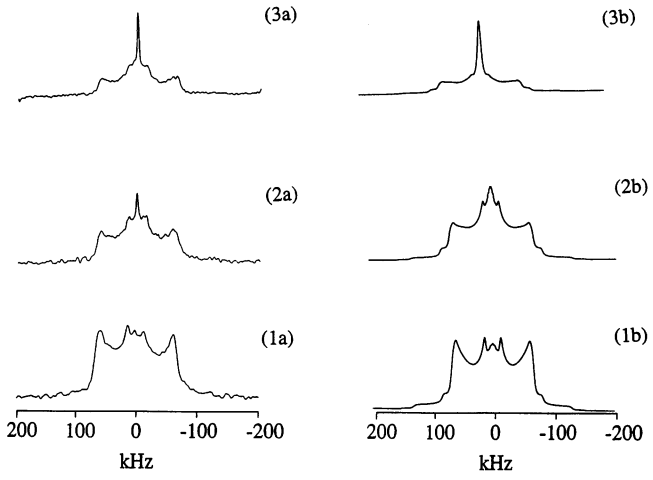


FIGURE 3 ^{2}H NMR spectra of polycrystalline carboxypeptidase A samples cross-linked with glutaraldehyde and soaked in a 10 mM solution of PPA-d5 obtained at 11.7 T at different water contents. The experimental conditions were the same as in Fig. 1. The corresponding simulated spectra were obtained using the approach described in Fig. 1, except that the quadrupole coupling constant was 175 kHz, $k_2 = 1.1 \times 10^4\ s^{-1}$ and (*1b*) $k_1 = 5 \times 10^5\ s^{-1}$, (*2b*) $k_1 = 2.5 \times 10^6\ s^{-1}$, and (*3b*) $k_1 = 4.0 \times 10^6\ s^{-1}$.

over the range of water contents studied, the rate constant is approximately an exponential function of the water activity.

The model used to simulate the spectra in Figs. 1–4 assumes that there is an exchange of the labeled molecule between the anisotropic bound environment and an isotropic unbound environment that is still inside the crystal. The spectra resulting from two-dimensional exchange experiments performed to test this assumption are shown in Figs. 6 and 7. In the case that the isotropic environment samples all of the bound anisotropic environments, cross-peak intensity may appear at all frequencies to be spanned by the powder pattern. The spectra do not have intensities significantly above the noise threshold in the wings of the powder pattern, although significant exchange is apparent in Fig. 7.

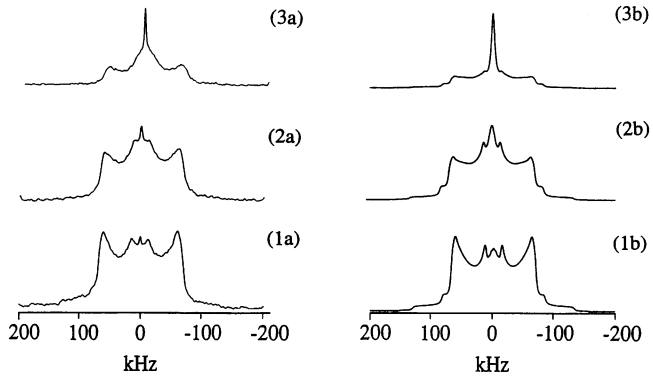


FIGURE 4 ^{2}H NMR spectra of polycrystalline carboxypeptidase A samples cross-linked with glutaraldehyde and soaked in a 10 mM solution of PAA-d5 obtained at 11.7 T at different water contents. The experimental conditions were same as in Fig. 1. The corresponding simulated spectra were obtained using the approach described in Fig. 3, except that $k_2 = 9.4 \times 10^3\ s^{-1}$

TABLE 1 Spectral dynamic parameters for carboxypeptidase A

Ligand	QCC (kHz)	a_w (%)	k_1 (s^{-1})	k_2 (s^{-1})	C_{ES}
L-Phenylalanine	180	33	1.9×10^5	1.6×10^4	0.9
		81	5.0×10^5	1.6×10^4	0.85
		98	7.5×10^5	1.6×10^4	0.7
D-Phenylalanine	180	33	1.9×10^5	1.1×10^4	0.9
		81	1.5×10^6	1.1×10^4	0.85
		98	1.9×10^6	1.1×10^4	0.7
Phenylpropionic acid	175	33	5.0×10^5	1.1×10^4	0.9
		81	2.5×10^6	1.1×10^4	0.85
		98	4.0×10^6	1.1×10^4	0.7
Phenylacetic acid	175	33	5.0×10^5	9.4×10^3	0.9
		81	2.5×10^6	9.4×10^3	0.85
		98	4.0×10^6	9.4×10^3	0.7

The exchange cross-peak intensity increases and then decreases with increases in the mixing time. When the crystals were equilibrated at 81% humidity, the maximum cross-peak intensity was near 1 ms; when equilibrated at 98% relative humidity, the maximum was near 0.7 ms.

Based on crystal structures, it is clear that for some bound molecules to move, the constraining environment must move as well. The importance of cooperative concerted motions of enzymes may be anticipated as a component of catalysis but is difficult to demonstrate experimentally. Phenylacetic acid crystals present an interesting and perhaps a limiting case of dynamical constraints imposed by molecular packing. This model differs fundamentally from the enzyme in that the local environment and the packing constraints are of high symmetry, which suggests that highly cooperative motions may be more probable. Nevertheless, this system presents interesting and surprising spectral features, in spite of its structural simplicity, that provide a calibration for more complex environments.

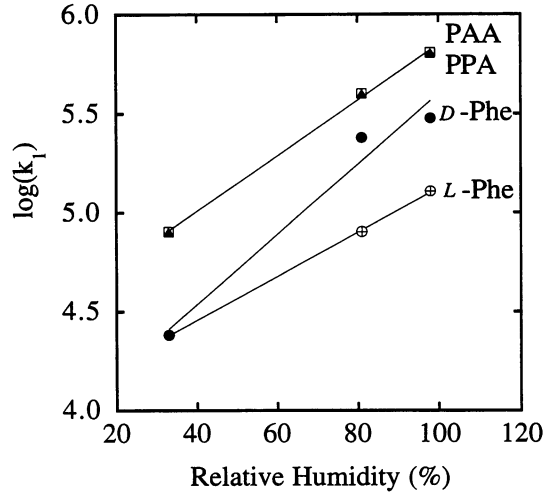
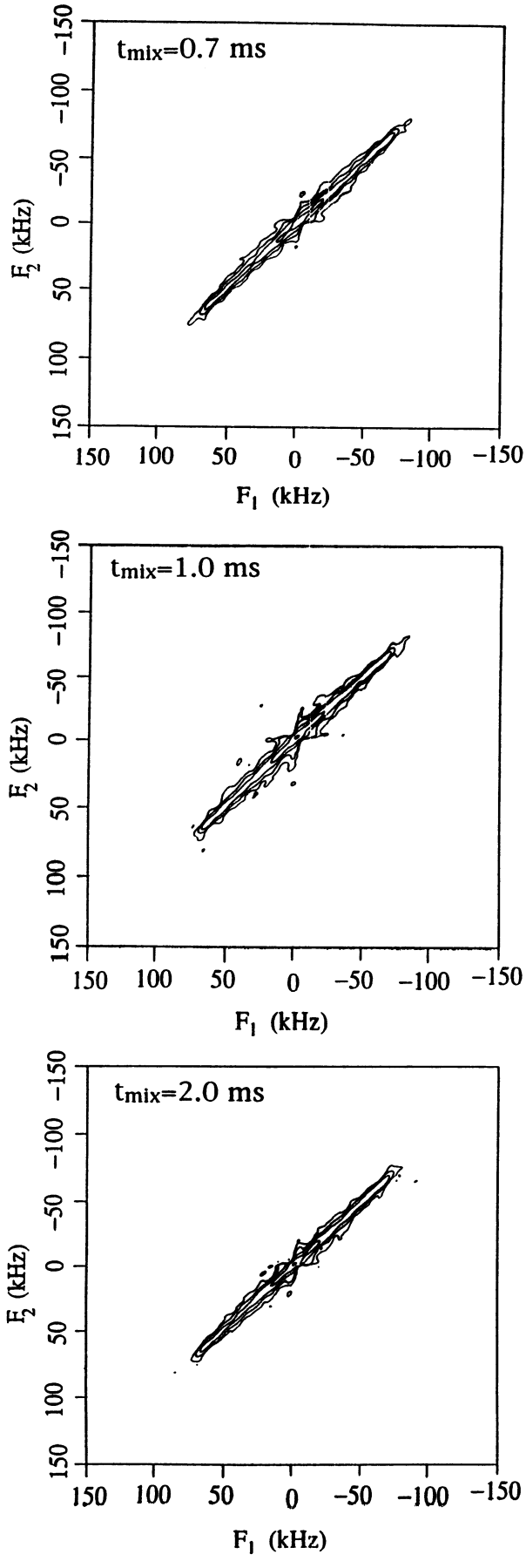


FIGURE 5 Logarithm of phenyl ring π-flip rates derived from the spectral modelling for carboxypeptidase A L-Phe-d5, D-Phe-d5, PPA-d5, and PAA-d5 bound to carboxypeptidase A versus relative humidity.



The deuterium spectra from the phenyl ring positions are shown in Fig. 8, and there are important spectral features at ±132.2, ±80.1, ±66.4, ±61.1, ±55.0, and ±13.6 kHz. The spectra were collected using a solid echo sequence, but the dependence on the echo delay over the range from 35 to 150 μs is not strong. The powder pattern does not change when the crystals are prepared by rapid thermal quenching or by slow recrystallization, and the x-ray powder diffraction patterns do not show significant differences. These powder pattern spectra cannot be explained without significant phenyl ring motion in the crystal. However, a single angular excursion may not account for the spectra. We may simulate these spectra as the superposition of three spectra corresponding to three jump angles of 24°, 32°, and 73°. The relative contributions of each are in the ratio of 1:1:2, and the rates for each jumping motion in the simulations were the same, 9.4×10^4 s^{-1}at 298 K. The powder patterns are shown in Fig. 9 for data taken at 315 K and the jumping rate required is 1.9×10^5 s^{-1}, but in this case as well, the spectra are simulated well if all three motions are assigned the same jump rate.

DISCUSSION

Bound ligand motions

Slight structural differences in either the ligand or the enzyme at the active binding site may produce substantial differences in binding affinity and local dynamics. In the previous study we developed the theoretical tools for examining local motions in carboxypeptidase A in the presence of ligand exchange between an anisotropic bound environment and isotropic unbound environments and demonstrated the approach using L-phenylalanine labeled with deuterium on the phenyl ring in carboxypeptidase crystals. We apply this model to the other optical isomer, which binds differently, and two carboxylic acids, phenylacetic acid and phenyl propionic acid, that differ in the distance between the carboxylic acid and the phenyl ring. The structure of D-phenyl alanine bound to carboxypeptidase A has been reported, based on x-ray diffraction studies (Christianson et al., 1989). The amide and carboxyl make linkages to Arg-145 and to Glu-270, whereas the phenyl ring is located in the P1′ region and is completely extended into the hydrophobic binding pocket of the protein. This pocket is bounded by α-helix on one side, an ω-shaped loop extending from residues 247 to 254 wrapping around the phenyl ring, and tyrosine-248 serving as a cap closing the pocket from the top (Lipscomb et al., 1970; Fetrow, 1995). Vallee and co-workers have examined PPA binding using carbon

FIGURE 6 Two-dimensional ^2H NMR exchange spectra for polycrystalline carboxypeptidase A samples cross-linked with glutaraldehyde and soaked in 10 mM PPA-d5 obtained at 11.7 T at a relative humidity of 81%. Spectra were obtained with approximately 200-mg samples, 8192 transients, a recycle delay of 0.5 s, an echo delay of 35 μs, and mixing times of (*a*) 0.7 ms, (*b*) 1 ms, and (*c*) 2 ms.

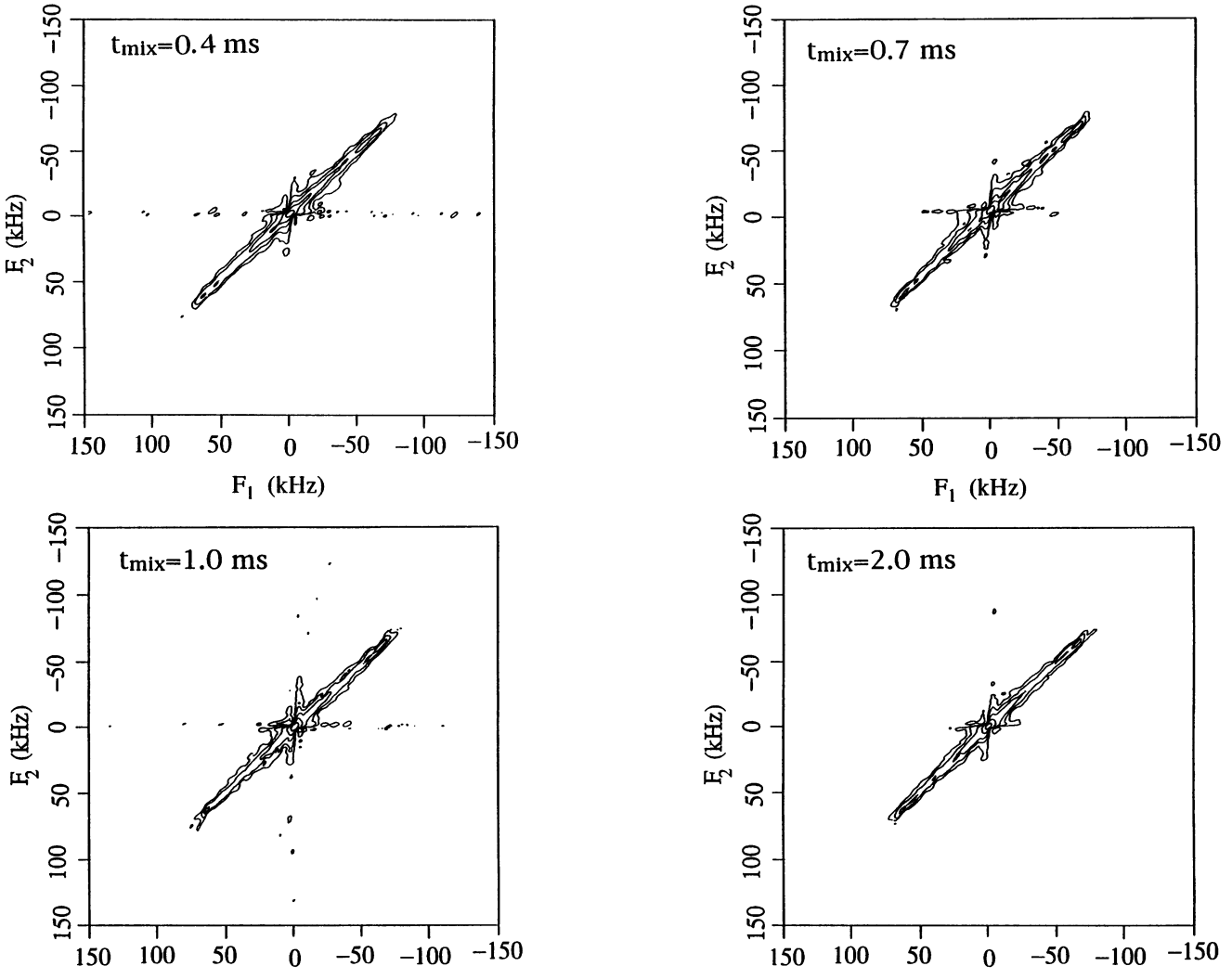


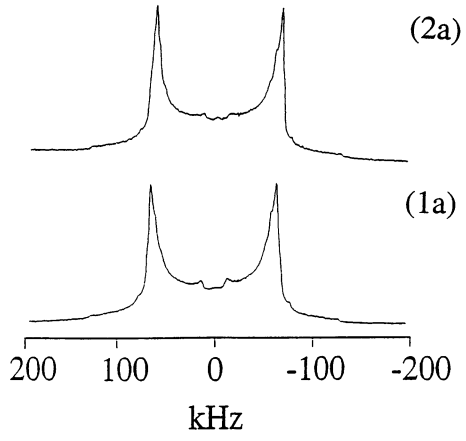
FIGURE 7 Two-dimensional ^2H NMR exchange spectra of polycrystalline carboxypeptidase A samples cross-linked with glutaraldehyde and soaked in 10 mM PPA-d5 obtained at 11.7 T at a relative humidity of 98%. The experimental conditions were the same as in Fig. 6, except that the mixing times were 0.4 ms, 0.7 ms, 1 ms, and 2 ms.

isotope labeling and have shown that PPA does not bind to the azoprotein, except at very high concentrations (Coleman and Vallee, 1964). The ^{35}Cl NMR studies of carboxypeptidase also suggest that the carboxylic acids like PPA bind directly to the zinc atom. The effect on the phenyl ring position is to pull the ring out of the hydrophobic pocket as the number of carbon atoms between the carboxylate and the phenyl ring is decreased.

The binding pocket loop for the phenyl ring is formed by Ser-254, Gly-253, Gly 252, Ser 251, Ala 250, Glu 249, Tyr-248, and Ile 247. The glycine peptide planes may rotate more easily than the rest, but all are susceptible to the effects of solvation and are stabilized less than residues in the β-sheet or α-helix portions of the protein. Therefore, changes in the solvent content may change the constraints provided on the motion of the bound ligand, particularly the rotationally labile phenyl ring portions of the ligand.

D- versus L-phenylalanine

D- and L-phenylalanine have binding modes similar to that of carboxypeptidase A in that the amine and carboxyl make salt links to Arg-145 and Glu-270. The phenyl ring inserts deeply into the hydrophobic pocket, although the chiral difference changes the orientation and the nonbonded interactions. The solution phase affinity is greater for D- than L-phenyl alanine, 2 mM versus 18 mM, respectively. The deuterium spectra for L-phenylalanine bound to carboxypeptidase A labeled in the ring positions are reproduced for convenience in Fig. 1. The deuterium spectra for D-phenylalanine shown in Fig. 2 have a splitting at ± 64.3 kHz and ± 15.6 kHz. The spectra also have distinguishable edges at ± 84 kHz at a relative humidity of 33%. These spectral features are signatures usually identified with π-flip motions of the ring when there is negligible libration of the rotation axis. We may fit the essential features of these



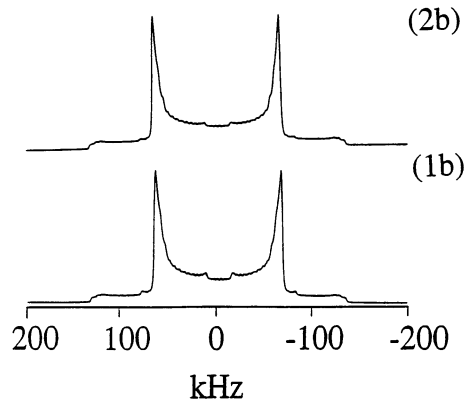
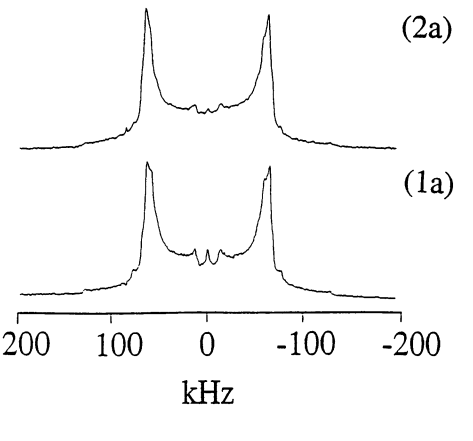


FIGURE 8 ^{2}H NMR spectra of phenylacetic acid recrystallized slowly and measured at 11.7 T at 298 K. Spectra were obtained using approximately 100-mg samples, 512 transients, and a recycle delay of 100 s. The deuterium quadrupole-echo delays were (*1a*) 35 μs, (*2a*) 150 μs. The corresponding simulated spectra (*1b*, *2b*) were obtained assuming three pairs of two-site jumping motions for the phenyl ring about the C-2 axis with the same jumping rate of 9.4×10^4 s^{-1}, a quadrupole coupling constant of 178 kHz, $\eta = 0.0$, line broadening of 1 kHz, and 2048 points in the spectral array. The three pairs of two sites have orientations with respect to the C-2 axis of the phenyl ring and populations, (0°, 60°, 0°) and (24°, 60°, 0°), $p_1 = p_2 = 0.5$; (0°, 60°, 0°) and (34°, 60°, 0°), $p_1 = p_2 = 0.5$; (0°, 60°, 0°) and (73°, 60°, 0°), $p_1 = p_2 = 0.5$. The spectral weight factors for the three pairs are 1:1:2.



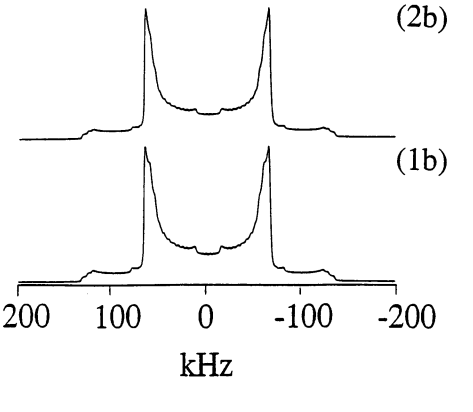


FIGURE 9 ^{2}H NMR spectra of polycrystalline phenylacetic acid recrystallized slowly, obtained at 11.7 T and 315 K. Experimental conditions were the same as summarized in Fig. 8, except that 256 transients were collected. The simulated spectra (*1b*, *2b*) were obtained with a ring jump rate of 1.9×10^5 s^{-1}.

spectra with an elementary model that includes a bound-state π-flip motion and an exchange between the bound anisotropic and unbound isotropic environments. The values of the quadrupole coupling constant and asymmetry parameter that fit the spectrum are 180 kHz and 0.05, respectively, which are reasonable values for phenyl ring deuterons. Examination of the x-ray structure using the Biosym program Insight II suggests that there should be sufficient space between the phenyl ring and the bounding residues of the protein pocket for the ring to execute the π-flip motions detected in the spectrum (Christianson et al., 1989; Burke et al., 1993; McCammon and Karplus, 1980; McCammon et al., 1979).

We note that this particular approach, which has been common in studies of polymer dynamics, does not represent a unique dynamical model for the motions in the complex system. The spectra are consistent with a family of similar solutions that may differ in the jump site populations and jump angles (Hirschinger and English, 1989). We use the simplest of these models, namely a two-site jump model with equal populations in which the phenyl ring executes an energetically degenerate π-flip or 180° sudden angular jump. Because the ligands are not covalently bonded to the protein and may have binding constants that leave some unbound ligand in equilibrium with bound ligand, the model includes the possibility of exchange between the bound and unbound environments. We assume that the resonance frequency for the ligand in the unbound environment is the isotropic value, because rapid motions in the interprotein spaces for molecules of this approximate size have been detected in protein crystals (Hsi et al., 1976). Furthermore, this model fits the spectra well.

It is interesting to note that the exchange rate required by the spectral simulation is smaller for D- than for L-phenylalanine, which is consistent with higher binding affinity for the D-isomer. However, the phenyl ring π-flip rates are 3–6 times larger for the D-isomer than the L-isomer, depending

on water content. Although the π-flip rates for both isomers increase with water content, the effects of water content are greater for the L-isomer, which is consistent with the motion of L-isomer being more constrained by contacts with the protein that change more with water content. When the water content is increased, the fraction of free ligand increases, which is reasonable because there is more water in which to dissolve it and the activity of the water increases to a level more nearly approximating that of the mother liquor (Burke et al., 1993).

Phenylacetic and propionic acids

Phenylpropionic acid has a binding constant of 62 μM, and although the main chain of the molecule is the same as phenylalanine, carboxyl coordination to the zinc moves the phenyl ring out of the hydrophobic pocket by approximately one carbon-carbon bond length. The spectra and simulations of the phenylpropionic acid in carboxypeptidase A crystals shown in Fig. 3 are substantially different from the phenylalanine spectra. At an a_w of 33%, the spectrum has singularities at $\pm$62 kHz, $\pm$13.6 kHz, and $\pm$80 kHz, which are consistent with a phenyl ring that undergoes a π-flip motion with a quadruple coupling constant that is 175 kHz and an asymmetry parameter of 0.05. A signal-to-noise ratio that is better for the phenylpropionic acid may be caused by a smaller value of k_2, the consequence of which is that the exchange event affects the rephasing of the solid echo less than in the more labile phenylalanine cases.

The reduced apparent quadrupole coupling constant required to simulate the spectra is a common feature of deuterium phenyl ring spectra when the π-flip axis is not stationary but may wobble. We assume that the coordination to the zinc atom fixes the carboxyl end of the bound molecule. Then the chain segment motions are rotation about either the C-C_α bond or the C_α-C_β bond. If we assume a two-site jumping model for the motion of the π-flip axis, and the jump rates are rapid, i.e., on the order of or greater than 10^7 s^{-1}, then the reduction in the quadrupole coupling constant is consistent with a high-frequency wobble of the π-flip axis of approximately $\pm$15°. This picture is oversimplified because there are two bonds about which rotations may occur that will reorient the π-flip axis in phenylpropionic acid; however, the basic result that additional small-angle high-frequency motions of the π-flip axis are required for the bound phenylpropionic acid will remain.

The other dramatic difference between phenylpropionic acid and the phenylalanines is the significantly increased sensitivity of the π-flip rate to hydration. The π-flip rate changes by a factor of 8 over the hydration range studied. Thus the ligand bound further outside the hydrophobic pocket reports greater dynamical changes as a function of hydration than the ligands with phenyl rings buried more deeply in the hydrophobic pocket.

Phenylacetic acid binds to the enzyme at the zinc atom with a dissociation constant of 390 μM, and the phenyl ring is pulled farther out of the hydrophobic pocket than in the phenyl propionic acid case. Nevertheless, the deuterium spectra and simulations are almost the same for phenylacetic acid and phenylpropionic acid. The spectral singularities for the 33% relative humidity sample at $\pm$62 kHz, $\pm$13.6 kHz, and $\pm$80 kHz are similar to PPA. The effective quadrupole coupling constant that is consistent with the spectra is 175 kHz, which may be interpreted in terms of a high-frequency π-flip axis wobble of $\pm$15°, which in this case must be about the C-C_α bond.

Water content dependence

The model applied demonstrates that the rate of bound ligand π-flip increases with water content for all ligands studied. Fig. 5 demonstrates that the dependence is approximately exponential in water activity, which is consistent with a correction to the activation barrier that is linear in the water activity over the range sampled. If we write

$$\Delta G^{\ddagger} = \Delta G^{\ddagger\circ} + b a_{w'} \tag{1}$$

then

$$\ln \frac{k_2}{k_1} = \frac{b}{RT}(a_{w1} - a_{w2}). \tag{2}$$

The value of $-b$ is 1.9 kcal/mol for PAA and PPA, and 1.3 kcal/mol for L-phenyl alanine, 2.1 kcal/mol for D-phenyl alanine. These numbers represent the change in the barrier for the π-flip motion caused by the addition of water to the cross-linked crystalline protein, and the negative sign indicates that the barrier is lower in the hydrated system. These estimates for the changes in barriers associated with hydration of the protein crystal system are small, but report changes in the motion that should be little influenced by solvent directly because the probe motion is a phenyl ring π-flip that responds to the available free volume or lack of intermolecular constraints in the binding pocket. Although the π-flip rates are slower, the barrier for the process changes less with water content when the phenyl ring is more deeply inserted in the hydrophobic pocket than when it is not. These results are qualitatively similar to the response of tyrosyl ring motions in α-lytic protease reported by Burke and co-workers (Burke et al., 1993).

Phenylacetic acid crystals

Phenylacetic acid is interesting because the molecular packing in the crystal is such that the overlap of the ring faces is approximately 80% and the interlayer spacing only 4.95 Å. If the van der Waals thickness of the ring is 3.4 Å, the remaining 1.5 Å does not leave enough space for free rotation of the phenyl rings. A partial reconstruction of the crystal packing is shown in Fig. 10 (Hodgson and Asplund, 1991). The phenyl ring deuterium spectra shown in Figs. 8 and 9 indicate that there is considerable phenyl ring motion in the crystal, even though the packing is tight. The spectra

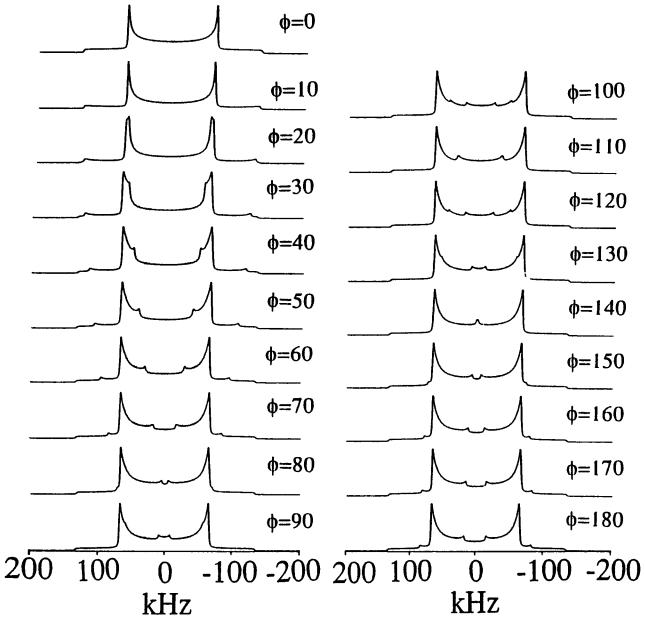


FIGURE 10 Molecular packing of phenylacetic acid in the crystal lattice according to the x-ray data obtained by Hodgson and Asplund. Only two molecules in the stack are shown.

are not consistent with complete π-flips such as those observed in carboxypeptidase A and many engineering polymers. However, the sharp edges shown may be reconstructed by assuming a superposition of phenyl ring two-site jump motions. Fig. 11 shows calculations of the powder pattern line shape for phenyl ring two-site jump motions

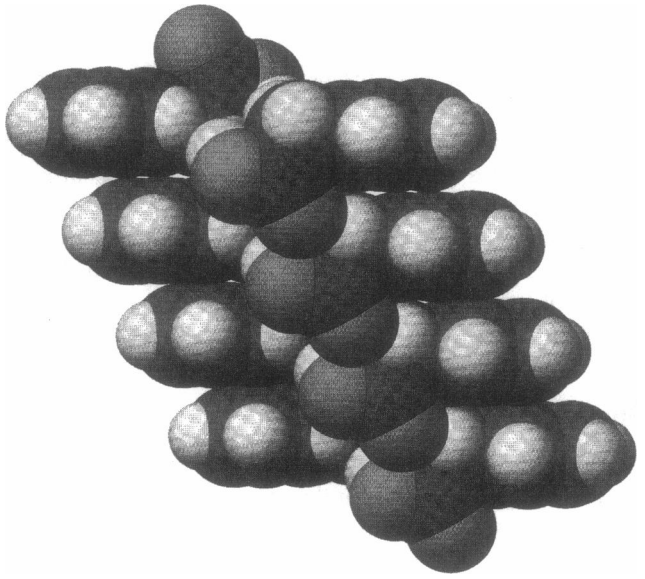


FIGURE 11 Simulated spectral patterns for two-site jumping model with equal site populations about the C-2 axis of the phenyl ring, assuming a quadrupole coupling constant of 178 kHz, η = 0.0, line broadening of 1 kHz, and 2048 points in the spectral array. The orientations of the two sites are (0°, 60°, 0°) and (ϕ, 60°, 0°), where the values of ϕ are shown in the figure.

about the C_2 axis for different choices of the jump angle Φ. Small but sharp features are clear for different choices of the Φ. The experimental spectra may be modeled well by a superposition of these spectra corresponding to jump angles of 24°, 32°, and 73°; relative populations of 1:1:2; and a common jump rate of 9.4×10^4 s^{-1}. Increasing the temperature increases the jumping rate that is needed to fit the spectrum, but the same rate satisfies all components of the spectrum.

A concern is that the motional differences that require a superposition are caused by defects in the crystal that permit different motions because the local packing is different. The x-ray powder patterns and the deuterium spectra are not different when the samples are recrystallized slowly or are thermally quenched. Although this does not rule out the possibility that there is a distribution of structure in the polycrystalline sample, it is unlikely that the defect populations could be large enough to contribute in the ratio of 1:1:2 to the total spectrum. The angular excursions required by the spectrum are large relative to the spaces available in the static crystal structure. The jump rates are not high (on the order of 10,000 s^{-1} at room temperature), so that there may be between 1 and 10 jumps during acquisition of the quadrupole solid echo. The simulation is a superposition of the spectra from these three angular excursions, but all of the phenyl rings in the crystal are equivalent. Thus the spectrum simulation requires that the mixing of these motions is slow compared with the acquisition time of the echo. This requirement means that the mixing or exchange between jump angles must be slow compared with a time on the order of a millisecond. Because the larger angle motions must involve cooperative displacement of neighboring rings in the solid, this does not appear to be an impossible requirement. If this simple model is appropriate as opposed to a different solution involving different probabilities, site lifetimes, or jump probabilities, then it would appear that the motions must be cooperative over a considerable distance.

The phenylacetic crystal was studied because it appears to represent a good model for motions in a highly constrained system. A relatively simple model may account for the spectra, although it raises interesting questions. For one molecule to reorient by an angle as large as 72°, the neighbor must move as well. Although cooperative motion appears inescapable, the exact character of the motion is not clear. We present a schematic representation of the motions in Fig. 12 that is consistent with the phenylacetic acid spectra of Figs. 8 and 9. This representation is not unique because the superposition of two-site jump models used for the spectral analysis is not unique, but this cartoon suggests that the motions must be locally coherent, or nearly so, for the sterically constrained rings to move. This is an attractive picture that appears to be relevant to critical motions in proteins, particularly such systems as the muscle proteins, in which motions eventually must be coherent to effect a contraction. However, these spectra by themselves do not provide proof that the motions are concerted or coherent,

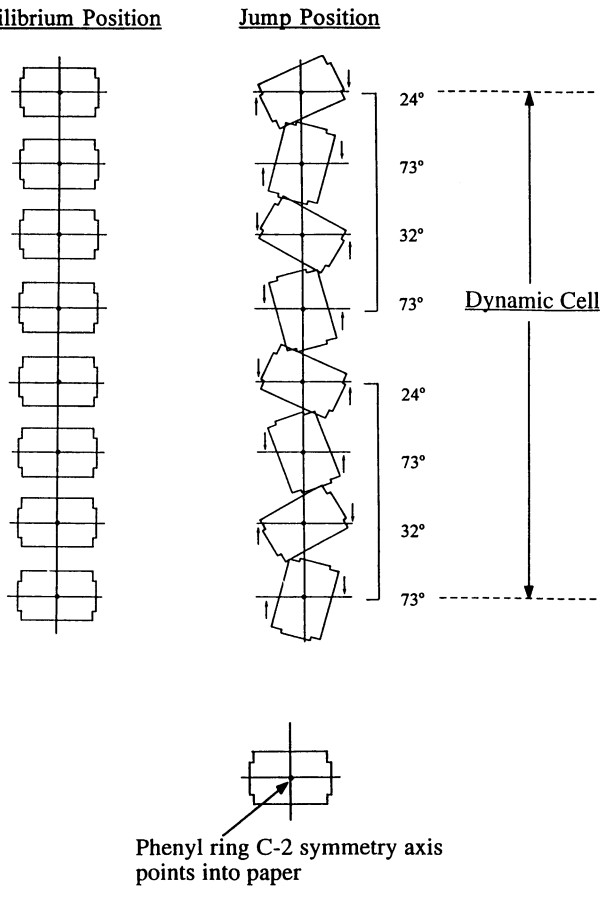


FIGURE 12 A representation of the highly cooperative phenyl ring motion in polycrystalline phenylacetic acid according to ^{2}H NMR powder spectral pattern analysis. The C-2 axis of the phenyl ring is perpendicular to the plane of the paper. Because of the symmetry of phenyl ring, the sign of the jump angle causes no difference in ^{2}H NMR spectra.

because the spectra and their analysis depend on the motion of only the deuterium-carbon bond, not on an interaction that depends on the relative position of two particles or rings simultaneously. These suggestions derive from the close molecular packing shown by the x-ray structure in combination with the dynamics implied by the deuterium NMR spectra.

CONCLUSION

The phenyl ring motions of the enzyme-bound ligands studied in the crystals of carboxypeptidase A are sensitive to the binding position and the water content of the crystals. The ring flip rates increase as the ring is withdrawn from the hydrophobic binding cave, and the rates become more sensitive to changes in the structure caused by hydration. The phenyl rings for all ligands studied have sufficient steric freedom in the bound environment to yield deuterium spectra that are reproduced well using a π-flip model that is distinct from the more highly constrained crystalline environment in phenylacetic acid crystals, where cooperative angular excursions of smaller amplitude are detected. The enzyme-bound dynamics of the ligand phenyl rings are sensitive to the hydration level of the cross-linked crystal system. A linear model for the water content dependence of the activation barrier yields contributions ranging from -1.3 to -2.1 kcal/mol, which are small and must reflect changes that derive from a number of competing contributions to the barrier for phenyl ring motions.

The authors gratefully acknowledge a number of conversations with many helpful scientists in the course of this work, including, in particular, Dr. P. Mark Henrichs, Professor Regitze Vold, Professor Jacob Schaefer, and Professor Richard Wittebort. We also thank Dr. Michal Sabat for assistance with the x-ray data.

This work was supported by the National Institutes of Health, 5R01GM34541, and the University of Virginia.

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